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# Signal Generation from Switchable Polydiacetylene Fluorescence

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## ABSTRACT

Chemical and biological sensors require a material component to act as a transducer from the molecular level event of interest to a discernable output measurable in the macroscopic world. One such material is polydiacetylene (PDA), a conjugated polymer that can switch from a non-emitting to a fluorescent state in response to environmental changes. This attribute can be harnessed to provide signal generation for bio-sensors and assays as a more sensitive alternative to the previously reported monitoring of PDA colorimetric shifts. While providing a more sensitive transduction mechanism the fluorescence behavior of PDA is also more complicated than the absorbance, in particular the emission profile of PDA in liposomes is strongly affected by the extent of polymerization. Incorporating small molecule fluorophores into the PDA materials further increases the overall emission of fluorescent PDA materials. The fluorophores accept energy from the excited state of the polymer and fluoresce, leading to both an overall increase in the quantum yield of the system and an increase in the Stokes shift. Basic photophysical properties of fatty acid PDA liposomes and a model assay for phospholipase $A_2$ are presented. The model assay results show that the fluorescence response is greater than the colorimetric, and is further enhanced by addition of fluorophores.

## INTRODUCTION

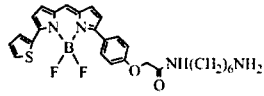
Liposomes containing polydiacetylene (PDA) can be used for colorimetric bio-assays in an approach that exploits the changeable absorbance properties of the conjugated polymer backbone of PDA and also takes advantage of the biomimetic aspect of surfactant liposomes. The liposomes are formed from diacetylene surfactants and the diacetylene tails are photopolymerized to form PDA in the alkyl regions of the liposomes. Changes in the conjugation state of the double and triple bonds of the PDA backbone due to external perturbations lead to shifts in the absorption maxima, typically causing a color change from blue to red. Charych et al photopolymerized diacetylene fatty acid liposomes with ligands or enzymatic substrates incorporated, to form PDA liposomes [1]. Exposure of the liposomes to solutions of a target analyte caused a gradual change in the color from blue to purple or red. Charych used PDA liposomes and films with sugars, gangliosides and phosphotidylcholines incorporated as colorimetric sensors for influenza virus [2], cholera toxin [3], and phospholipases [4]. Subsequently, other researchers have developed similar colorimetric assays using PDA liposomes and films [5]. For all these colorimetric assays the liposomes or films were prepared in the blue state and upon interaction with the analyte turned red. The change in the absorption properties is quantified by calculation of the "colorimetric ratio" (CR); the ratio of the intensity of the "blue" maximum absorbance peak (650nm) over the sum of the intensities of the "blue" and "red" (550 or 490nm) absorbance peaks.

The work presented here uses the changeable fluorescence of PDA rather than the colorimetric properties for signal transduction [6]. The emissive properties of PDA are also

dependent on the conjugation length of the backbone; PDA assemblies with long conjugation lengths (i.e. blue PDA) are non-fluorescent. If the conjugation lengths are shortened (i.e. red or yellow PDA) the materials become fluorescent. The fluorescence of red PDA and the non-fluorescence of blue PDA is well established and has been noted in liposomes [7], however, the ability of PDA to change from non-fluorescent to fluorescent has not been previously exploited for sensing or assay development purposes to any significant extent. A model assay based on the colorimetric assay for phospholipase $A_2$ ($PLA_2$) developed by Charych et al [4] was run for the purpose of comparing the colorimetric response to the fluorescence response.

## EXPERIMENTAL DETAILS

10,12-pentacosadiynoic acid (PCDA) and 10,12-tricosadiynoic acid (TRCDA) were purchased from GFS and recrystallized once from hexane. Dimyristoyl phopsphotidylcholine (DMPC) and phospholipase $A_2$ ($PLA_2$) were from Sigma; buffer salts and solvents were from Fisher. Organic fluorophores, 5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-31,41-diaza-*s*-indacene-3-yl)phenoxy)acetyl)amino)pentylamine hydrochloride (**1**) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate salt (**2**), **Figure 1**, were obtained from Molecular Probes and incorporated at a ratio of 1 fluorophore:200 surfactants. $H_2O$ was purified by ultra-filtration through a Millipore Milli-Q Plus system and dispensed with a resistance of 18.2 MΩcm. Photopolymerization was performed in a Fisherbrand cross-linking oven capable of delivering controlled energy doses of UV light at 254nm.

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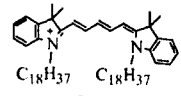


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**Figure 1**. Fluorophores **1** and **2**

### Polymerization and Emission

TRCDA liposomes and TRCDA liposomes with fluorophores **1** and **2** were prepared by sonication of surfactants in water, chilled (10 °C) and polymerized with 200mJ/cm$^2$ of irradiation to give deep blue solutions [6]. PCDA liposomes with and without fluorophores **1** and **2** were prepared and individual 100μL portions were polymerized with increasing doses of UV at 254nm, also giving blue solutions. The polymerized liposome solutions were then diluted 1:9 with water and heated at 70 °C for 15m; the heating step converts the PDA irreversibly to the red fluorescent form [1]. The emission and absorbance spectra of the heated liposomes were collected and the ratios of the intensities of the fluorophore fluorescence peaks (630nm for **1** and 665nm for **2**) to the PDA fluorescence peak at 560nm were calculated.

### Phospholipase $A_2$ Assay [4]

Liposomes were prepared from a mixture of 10,12-tricosadiynoic acid (TRCDA) and dimyristoyl phosphatidylcholine (DMPC) in a 7:2 molar ratio with and without fluorophore **1**. The liposomes were polymerized with 200mJ/cm$^2$ of UV energy at 254nm forming blue solutions. Phospholipase $A_2$ ($PLA_2$) was dissolved in water at 1.0mg/mL. A mixed buffer solution was

prepared of TRIS (50mM) and NaCl (150mM) at pH 7.7 and combined with the $PLA_2$ solution at 9 parts buffer to 1 part $PLA_2$ solution. This solution was added to the liposome solution, diluting it to 0.091mM in liposomes. A control sample (CTRL) was prepared using 9 parts mixed buffer to 1 part $H_2O$ in lieu of the $PLA_2$ solution. The fluorescence of the samples was read at intervals over 30m. Photobleaching of the liposomes during the assay was not observed. An equivalent assay was run with the absorbance at 650nm and 550nm measured. The colorimetric ratio (CR) was calculated at each time point and the percentage changes from the initial values of the CR and of the emission were also calculated. It should be noted that **1** has negligible absorbance at both 650nm and 550nm.

## RESULTS AND DISCUSSION

Both fluorescent poly(PCDA) and poly(TRCDA) liposomes have two emission maxima, however, the relative intensities are different as can be seen in **Figure 2**. It was hypothesized that relative intensities are dependent upon the extent of polymerization of the diacetylene in the liposomes, with the peak at 550nm dominating when the PDA chains are few and relatively isolated and the 625nm peak increasing as the amount of polymer increases. TRCDA liposomes are more readily polymerized than PCDA liposomes and hence have a higher density of polymer chains. After a UV dose of 200mJ/cm$^2$ the heated poly(TRCDA) liposomes have an absorbance at 550nm of 0.35 while the heated poly(PCDA) liposome absorbance is only 0.06. Changes in emission profile with inter-chain distance and/or extent of polymerization are precedented in other fluorescent polymer systems [8].

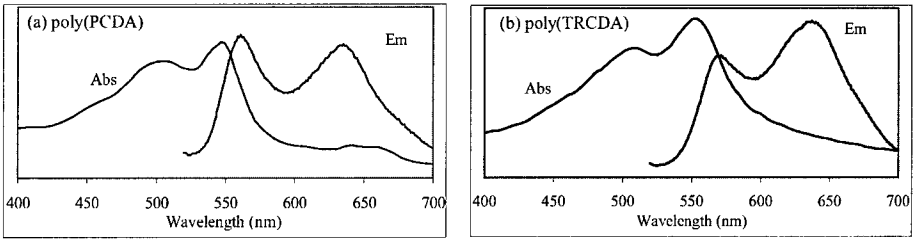


**Figure 2**. Absorbance (Abs) and emission (Em) spectra (arbitrary units) for (a) poly(PCDA) liposomes (heated) and (b) poly(TRCDA) liposomes (heated).

A study was performed to examine the effect of UV dose (and hence polymerization extent) on the emission profile of poly(PCDA) liposomes. After exposure to low UV doses, with the PDA chains surrounded by monomeric diacetylenes, the first emission peak of the liposomes (560nm) dominated; as the overall extent of polymerization increased the second emission peak (620nm) grew in. The absorbance spectra of the fluorescent liposomes also had two peaks at 550nm and 500nm, however, the relative intensities remained similar even as the overall absorbance increased with further polymerization (**Figure 3**). The differing effects of increased polymerization on the profiles of the absorption and emission spectra suggested that the increase in the 625nm PDA emission peak with higher UV doses did not simply arise from an increase of PDA chains with a specific conjugation length leading to the 625 emission peak, but from interactions of multiple PDA chains. This is consistent with earlier studies performed on PDA

chains in solution: fully solubilized PDA had only one emission peak while aggregated or colloidal PDA showed two emission peaks [9].

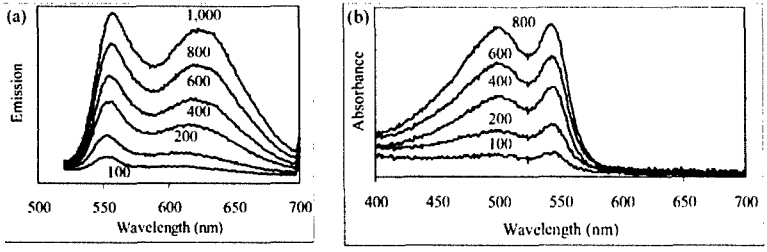


**Figure 3**. (a) Emission (Em.) and (b) absorbance spectra for fluorescent poly(PCDA) liposomes, (arbitrary units) at UV irradiation doses of 100, 200, 400, 600, 800 and 1000 (Em. only) mJ/cm$^2$.

Fluorescent polymers often have low quantum yields because the conjugated chains can act as excited state traps and provide mechanisms for non-radiative relaxation of the excited state, leading to quenching of the fluorescence [10]. Lipophilic fluorophores **1** and **2** were incorporated in the liposomes. The excitation spectra of these fluorophores overlapped with the emission spectrum of fluorescent PDA. When the liposomes, in the fluorescent state, were exposed to a wavelength that excited **only** the PDA the majority of the emission occurred at the fluorophore's emission wavelength and the overall emission was much higher than for equivalent PDA liposomes without the fluorophores (**Figure 4**). The overall rise in the emission suggested that the energy transfer from the PDA to the fluorophores competed with non-radiative decay of the PDA excited state. It should be noted that for liposomes with **1** the fluorophore emission and the 625nm emission peak of PDA overlapped, however, the fluorophore emission was much greater than the PDA emission (**Figure 4a**). The addition of the fluorophores to the PDA liposomes did not make the liposomes fluorescent when the PDA chains were in the non-fluorescent, blue, state. The emission profile of the fluorescent PDA liposomes affected whether the PDA could transfer energy to incorporated fluorophores. Poly(PCDA) underwent energy transfer with several fluorophores including both **1** and **2**, while poly(TRCDA) liposomes underwent effective energy transfer only with **1**. A second practical benefit from incorporating fluorophores in the PDA liposomes was that monitoring the emission of the system at the fluorophores' maximum emission during assays increased the effective Stokes shift of the system. Increase of the Stokes shift reduced the background fluorescence caused by reflection and scatter of the excitation light.

It was noted in early experiments that the extent of energy transfer seemed to decrease when larger UV doses were used for polymerization of the liposomes. This prompted a study on the effect of polymerization energy on energy transfer efficiency with PCDA liposomes incorporating **1** and **2**. As seen in **Figure 4(b)**, at first the fluorophore emission increased relative to the PDA emission 560nm as the diacetylene/fluorophore liposomes were exposed to increasing doses of UV. Above a UV dose of 20-30mJ/cm$^2$, however, the fluorophore emission decreased relative to the emission of the PDA. This suggested that as the population of PDA chains in the liposomes increased inter-chain interactions competed with energy transfer to the fluorophores. When preparing PDA/fluorophore liposomes for use in assays a balance needed to be struck between increasing the extent of polymerization to improve the stability of the

liposomes and keeping the extent of polymerization low to optimize the energy transfer to the incorporated fluorophore.

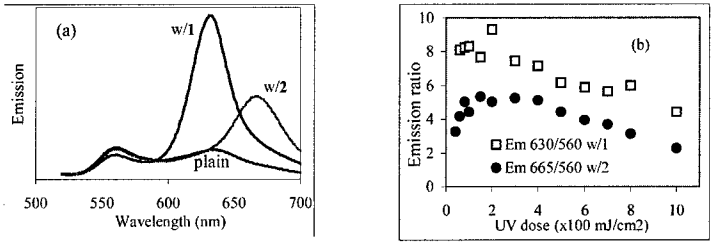


**Figure 4.** (a) Emission spectra of fluorescent poly(PCDA) liposomes, plain and with **1** and **2**. (b) Ratios of **1** and **2** emission (Em) (630nm and 665nm respectively) to PDA emission at 560nm vs. UV dose.

## Example Assay

The same general PDA liposome formulations that have been used for colorimetric bio-sensing can also be used for fluorescence based sensing [1]. An assay for $PLA_2$ activity based on a literature assay [4] using poly(TRCDA)/DMPC liposomes and poly(TRCDA)/DMPC liposomes with **1** incorporated was run and monitored by emission and absorbance. The incorporation of **1** for signal enhancement did not appear to appreciably affect the stability of the liposomes. The assay results, presented as absolute percent change from the initial values of the emission and the CR, are shown in **Figure 5**. It can be seen that the overall changes in the emission were greater than the changes of the CR and that the distinction between the $PLA_2$ sample and the control sample was also greater. Addition of **1** also increased the change in the emission, while not significantly affecting the CR. Comparison of the slopes of the $PLA_2$ data and the control data indicated that the majority of the $PLA_2$ activity occurred within the first 10m of the assay and the changes thereafter were from the action of the buffer on the liposomes. The trend and the extent of the change in CR were consistent with the results previously obtained by Charych et al. Assays run with poly(PCDA)/DMPC liposomes gave similar results although the percent changes in emission and CR are both lower. Subsequent work on other assays has shown that changing to a lower ionic strength buffer and using a different buffer salt can reduce the background activity of the control sample.

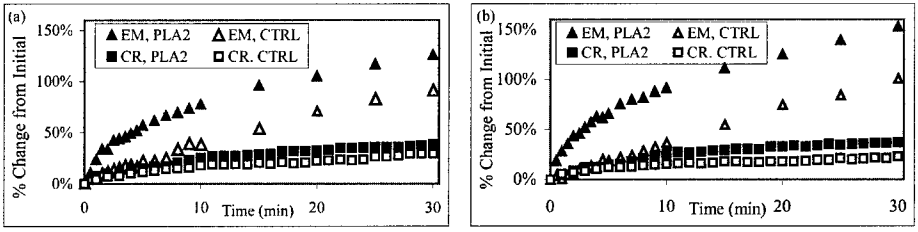


**Figure 5.** Percent change relative to initial values of emission (Em) and CR values of $PLA_2$ samples and buffer control samples versus time for the $PLA_2$ assay with (a) poly(TRCDA)/DMPC liposomes, and (b) poly(TRCDA)/DMPC liposomes with **1**.

## Conclusions

The conversion of polydiacetylene (PDA) in liposomes from non-fluorescent to fluorescent can be exploited for signal generation in bio-assays. The changes in the emissive and absorbance properties of the PDA liposomes arise from changes in the conjugation length of the PDA backbone $\pi$ bonds, however, the emissive behavior is more complex and appears to be affected by interactions between polymer chains. The emission of fluorescent PDA liposomes can be enhanced by addition of fluorophores that participate in energy transfer from the excited PDA to the fluorophore. A comparison of equivalent emission and absorbance assays for $PLA_2$ with polymerized diacetylene fatty acid/DMPC liposomes showed that the change in emission of the liposomes during the assay was greater than the change in absorbance. Using liposomes with a fluorophore incorporated in the assay further increased the change in emission in response to $PLA_2$. The results from these model assays suggest that fluorescent assays with PDA liposomes may allow detection of analytes at lower levels than the previously described colorimetric assays.

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## REFERENCES

1. Okada S., Peng S., Spevak W., Charych D. *Acc. Chem. Res.* **1998**, *31*, 229-239.
2. Spevak W., Nagy J. Charych D. *Adv. Mater.* **1995**, *7(1)*, 85-89.
3. Pan J., Charych D. *Langmuir* **1997**, *13*, 1365-1367.
4. Okada S., Jelinek R., Charych D. *Angew. Chem. Int. Ed Eng.* **1998**, *38*, 655-659.
5. (a) Cheng Q. and Stevens R. C., *Adv. Mater.* **1997** *9(6)*, 481-483. (b) Ma Z., Li J., Liu M. Cao J., Zou Z., Tu J., Jiang L. *J. Am. Chem. Soc.* **1998**, *120*, 12678-12679. (c) Kolushcva S., Shahal T., Jelinek R. *J. Am. Chem. Soc.* **2000**, *122*, 776-780. (d) Kolusheva S., Boyer L., Jelinek R. *Nat. Biotechnol.* **2000**, *18*, 225-227. (e) Kolusheva S., Kafri R., Marina K., Jelinek R. *J. Am. Chem. Soc.* **2001**, *123*, 417-422.
6. Reppy M. A., Sporn S. A., Saller C. F., "Method for detecting an Analyte by Fluorescence", PCT International Patent WO/00171317 (March 20, 2001).
7. Singh A., Thompson R.B., Schnur J.M. *J. Am. Chem. Soc.* **1986**, *108*, 2785-2787.
8 . (a) Bazan G. C., Miao Y-J; Renak M. L., Sun B. J. *J. Am. Chem. Soc.* **1996**, *118*, 2618-2624. (b) McQuade D. T., Kim J., Swager T. M. *J. Am. Chem. Soc.* **2000**, *122*, 5885-5886.
9 . (a) Bloor D., Batchelder D.N., Ando D. J. *J. Polym. Sci.* **1981**, *19*, 321-334. (b) Kotaka T., Ohnuma H. *Chem. Phys. Lett.* **1985**, *114*, 446-450. (c) Rumbles G., Brown A. J., Phillips D., Bloor D. *J. Chem. Soc. Faraday Trans.* **1992**, *88(22)*, 3313-3318.
10. Barashkov N.N.; Gunder O.A. *Fluorescent Polymers*; Ellis Horwood: New York, 1994; Chapter 1.